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(21) International Application Number: PCT/DK99/00628 (22) International Filing Date: 16 November 1999 (16.11.99) (30) Priority Data: PA 1998 01495 16 November 1998 (16.11.98) DK (71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors: SVENDSEN, Allan; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). KJÆRULFF, Søren; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). BISGÅRD-FRANTZEN, Henrik; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). ANDERSEN, Carsten; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: α -AMYLASE VARIANTS (57) Abstract The invention relates to a variant of a parent Termamyl-like α -amylase, comprising mutations in two, three, four, five or six regions/positions. The variants have increased stability at high temperatures (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an α -amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an α -amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an α -amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an α -amylase variant of the invention, a method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased.		

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Title: α -amylase variants

FIELD OF THE INVENTION

The present invention relates to novel variants of parent
5 Termamyl-like α -amylases with altered properties relative of the
parent alpha-amylase. Said properties include increased
stability, e.g., at acidic pH, e.g., at low calcium
concentrations and/or high temperatures. Such variants are
suitable for a number of applications, in particular, industrial
10 starch processing (e.g., starch liquefaction or
saccharification).

BACKGROUND OF THE INVENTION

α -Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1)
15 constitute a group of enzymes which catalyze hydrolysis of
starch and other linear and branched 1,4-glucosidic oligo- and
polysaccharides.

There is a very extensive body of patent and scientific
literature relating to this industrially very important class of
20 enzymes. A number of α -amylase such as Termamyl-like α -
amylases variants are known from, e.g., WO 90/11352, WO
95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

WO 96/23874 provides the three-dimensional, X-ray crystal
structural data for a Termamyl-like α -amylase which consists of
25 the 300 N-terminal amino acid residues of the *B.*
amyloliquefaciens α -amylase and amino acids 301-483 of the C-
terminal end of the *B. licheniformis* α -amylase comprising the
amino acid sequence (the latter being available commercially
under the tradename Termamyl™), and which is thus closely
30 related to the industrially important *Bacillus* α -amylases (which
in the present context are embraced within the meaning of the
term "Termamyl-like α -amylases", and which include, *inter alia*,
the *B. licheniformis*, *B. amyloliquefaciens* and *B.*
stearothermophilus α -amylases). WO 96/23874 further describes
35 methodology for designing, on the basis of an analysis of the

structure of a parent Termamyl-like α -amylase, variants of the parent Termamyl-like α -amylase which exhibit altered properties relative to the parent.

5 BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel α -amylolytic variants (mutants) of a Termamyl-like α -amylase, in particular variants exhibiting increased stability at acidic pH at high temperatures (relative to the parent) which are advantageous in
10 connection with, e.g., the industrial processing of starch (starch liquefaction, saccharification and the like) as described in US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

15 Starch conversion

A "traditional" starch conversion process degrading starch to lower molecular weight carbohydrate components such as sugars or fat replacers includes a debranching step.

20 "Starch to sugar" conversion

In the case of converting starch into a sugar the starch is depolymerized. A such depolymerization process consists of a pretreatment step and two or three consecutive process steps, viz. a liquefaction process, a saccharification process and
25 dependent on the desired end product optionally an isomerization process.

Pre-treatment of native starch

Native starch consists of microscopic granules which are
30 insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typically
35 industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today

mostly obtained by enzymatic degradation.

Liquefaction

During the liquefaction step, the long chained starch is
5 degraded into branched and linear shorter units (maltodextrins)
by an α -amylase (e.g., Termamyl™ SEQ ID NO: 4 herein). The
liquefaction process is carried out at 105-110°C for 5 to 10
minutes followed by 1-2 hours at 95°C. The pH lies between 5.5
and 6.2. In order to ensure an optimal enzyme stability under
10 these conditions, 1 mM of calcium is added (40 ppm free calcium
ions). After this treatment the liquefied starch will have a
"dextrose equivalent" (DE) of 10-15.

Saccharification

15 After the liquefaction process the maltodextrins are
converted into dextrose by addition of a glucoamylase (e.g.,
AMG™) and a debranching enzyme, such as an isoamylase (US
Patent 4,335,208) or a pullulanase (e.g., Promozyme™) (US
Patent
20 4,560,651). Before this step the pH is reduced to a value below
4.5, maintaining the high temperature (above 95°C) to
inactivate the liquefying α -amylase to reduce the formation of
short oligosaccharide called "panose precursors" which cannot
be hydrolyzed properly by the debranching enzyme.

25 The temperature is lowered to 60°C, and glucoamylase and
debranching enzyme are added. The saccharification process
proceeds for 24-72 hours.

Normally, when denaturing the α -amylase after the
liquefaction step about 0.2-0.5% of the saccharification
30 product is the branched trisaccharide 6²- α -glucosyl maltose
(panose) which cannot be degraded by a pullulanase. If active
amylase from the liquefaction step is present during
saccharification (i.e., no denaturing), this level can be as
high as 1-2%, which is highly undesirable as it lowers the
35 saccharification yield significantly.

Isomerization

When the desired final sugar product is e.g. high fructose syrup the dextrose syrup may be converted into fructose.

- 5 After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucoseisomerase (such as Sweetzyme™).

10

In the context of the invention the term "acidic pH" means a pH below 7.0, especially below the pH range in which industrial starch liquefaction processes are traditionally performed, as described above, which is between pH 5.5 and 6.2.

- 15 In the context of the present invention the term "low Calcium concentration" means concentrations below the normal level used in traditional industrial starch liquefaction processes, such as between 0-40 ppm, preferably between 10-30 ppm, such as between 15-25 ppm Calcium. Normal concentrations
20 vary depending of the concentration of free Ca^{2+} in the corn. Normally a dosage corresponding to 1mM (40ppm) is added which together with the level in corn gives between 40 and 60 ppm free Ca^{2+} .

- In the context of the invention the term "high temperature"
25 means temperatures between 95 and 160°C, especially the temperature range in which industrial starch liquefaction processes are normally performed, which is between 95 and 105°C.

- The invention further relates to DNA constructs encoding
30 variants of the invention, to methods for preparing variants of the invention, and to the use of variants of the invention, alone or in combination with other α -amylolytic enzymes, in various industrial processes, in particular starch liquefaction.

35 Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used.

For ease of reference, α -amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ (A30-N33).

Where a specific α -amylase contains a "deletion" in comparison with other α -amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively. Multiple mutation may also be separated as follows, i.e., meaning the same as the plus sign:

Ala30Asp/Glu34Ser or A30N/E34S

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine

in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

5

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α -amylases in the context of the invention. The numbers on the Extreme left designate the respective amino acid sequences as follows:

1: SEQ ID NO: 2,

2: amylase

3: SEQ ID NO: 1,

4: SEQ ID NO: 5,

15 5: SEQ ID NO: 4,

6: SEQ ID NO: 3.

Figure 2 shows the PCR strategy used in Example 1.

20 DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α -amylase

It is well known that a number of α -amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 4 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquefaciens* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 5 and about 79% homologous with the *B. stearothermophilus* α -amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α -amylases include an α -amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α -amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

Still further homologous α -amylases include the α -amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α -amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α -amylases are Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

Because of the substantial homology found between these α -amylases, they are considered to belong to the same class of α -amylases, namely the class of "Termamyl-like α -amylases".

Accordingly, in the present context, the term "Termamyl-like α -amylase" is intended to indicate an α -amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e., the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4 herein. In other words, a Termamyl-like α -amylase is an α -amylase which has the amino acid sequence shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) or i) which displays at least 60% homology (identity), preferred at least 70%, more preferred at least 75%, even more preferred at least 80%, especially at least 85%, especially preferred at least 90%, especially at least 95%, even especially more preferred at least 97%, especially at least 99% homology with at least one of said amino acid sequences shown in SEQ ID NOS 1: or 2 or 3 or 4 or 5 or 6 or 7 or 8 and/or ii) displays immunological cross-reactivity with an antibody raised against one or more of said α -amylases, and/or iii) is encoded by a DNA sequence which hybridizes, under the low to very high stringency conditions (said conditions described below) to the

DNA sequences encoding the above-specified α -amylases which are apparent from SEQ ID NOS: 9, 10, 11, 12, and 32, respectively, of the present application (which encodes the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4, and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

10 In connection with property i), the "homology" (identity) may be determined by use of any conventional algorithm, preferably by use of the gap programme from the GCG package version 8 (August 1994) using default values for gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of
15 0.1 (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 8, 575 Science Drive, Madison, Wisconsin, USA 53711).

The parent Termamyl-like α -amylase backbone may in an embodiment have an amino acid sequence which has a degree of
20 identity to SEQ ID NO: 4 of at least 65%, preferably at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 97%, and even more preferably at least 99% identity
25 determined as described above

A structural alignment between Termamyl® (SEQ ID NO: 4) and a Termamyl-like α -amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α -amylases. One method of obtaining said structural alignment is
30 to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading
35 (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998)).

For example, the corresponding positions, of target residues found in the C-domain of the *B. licheniformis* α -amylase, in the amino acid sequences of a number of Termamyl-like α -amylases which have already been mentioned are as follows:

5											
	Termamyl-like α -amylase										
10	<table> <tr> <td data-bbox="279 730 909 758"><i>B. lich.</i> (SEQ ID NO: 4)</td><td data-bbox="909 730 1378 758">S356 Y358 E376 S417 A420</td></tr> <tr> <td data-bbox="279 772 909 800"><i>B. amylo.</i> (SEQ ID NO: 5)</td><td data-bbox="909 772 1378 800">S356 Y358 E376 S417 A420</td></tr> <tr> <td data-bbox="279 814 909 842"><i>B. stearo.</i> (SEQ ID NO: 3)</td><td data-bbox="909 814 1378 842">---- Y361 ---- ---- ----</td></tr> <tr> <td data-bbox="279 856 909 884"><i>Bac.WO 95/26397</i> (SEQ ID NO: 2)</td><td data-bbox="909 856 1378 884">---- Y363 ---- S419 ----</td></tr> <tr> <td data-bbox="279 898 909 926"><i>Bac.WO 95/26397</i> (SEQ ID NO: 1)</td><td data-bbox="909 898 1378 926">---- Y363 ---- ---- ----</td></tr> </table>	<i>B. lich.</i> (SEQ ID NO: 4)	S356 Y358 E376 S417 A420	<i>B. amylo.</i> (SEQ ID NO: 5)	S356 Y358 E376 S417 A420	<i>B. stearo.</i> (SEQ ID NO: 3)	---- Y361 ---- ---- ----	<i>Bac.WO 95/26397</i> (SEQ ID NO: 2)	---- Y363 ---- S419 ----	<i>Bac.WO 95/26397</i> (SEQ ID NO: 1)	---- Y363 ---- ---- ----
<i>B. lich.</i> (SEQ ID NO: 4)	S356 Y358 E376 S417 A420										
<i>B. amylo.</i> (SEQ ID NO: 5)	S356 Y358 E376 S417 A420										
<i>B. stearo.</i> (SEQ ID NO: 3)	---- Y361 ---- ---- ----										
<i>Bac.WO 95/26397</i> (SEQ ID NO: 2)	---- Y363 ---- S419 ----										
<i>Bac.WO 95/26397</i> (SEQ ID NO: 1)	---- Y363 ---- ---- ----										
15											

As will be described further below mutations of these conserved amino acid residues are very important in relation to increasing the stability at acidic pH and/or at low calcium concentration at high temperatures.

Property ii) (see above) of the α -amylase, i.e., the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α -amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g., as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g., as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α -amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8 respectively, have been found.

The oligonucleotide probe used in the characterization of the Termamyl-like α -amylase in accordance with property iii)

above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α -amylase in question.

Suitable conditions for testing hybridization involve
5 presoaking in 5xSSC and prehybridizing for 1 hour at -40°C in a
 solution of 20% formamide, 5xDenhardt's solution, 50mM sodium
 phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus
 DNA, followed by hybridization in the same solution supplemented
 with 100mM ATP for 18 hours at -40°C , followed by three times
10 washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes
 (low stringency), preferred at 50°C (medium stringency), more
 preferably at 65°C (high stringency), even more preferably at
 -75°C (very high stringency). More details about the
 hybridization method can be found in Sambrook et al., Molecular
15 Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only
to indicate an α -amylase produced or producible by a strain of
the organism in question, but also an α -amylase encoded by a DNA
sequence isolated from such strain and produced in a host or-
20 ganism transformed with said DNA sequence. Finally, the term is
intended to indicate an α -amylase which is encoded by a DNA
sequence of synthetic and/or cDNA origin and which has the
identifying characteristics of the α -amylase in question. The
term is also intended to indicate that the parent α -amylase may
25 be a variant of a naturally occurring α -amylase, i.e., a variant
which is the result of a modification (insertion, substitution,
deletion) of one or more amino acid residues of the naturally
occurring α -amylase.

30 Parent hybrid α -amylases

The parent α -amylase (backbone) may be a hybrid α -amylase,
i.e., an α -amylase which comprises a combination of partial
amino acid sequences derived from at least two α -amylases.

The parent hybrid α -amylase may be one which on the basis of

amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α -amylase family. In this case, the hybrid α -amylase is typically composed of at least one part of a
5 Termamyl-like α -amylase and part(s) of one or more other α -amylases selected from Termamyl-like α -amylases or non-Termamyl-like α -amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α -amylase may comprise a combination
10 of partial amino acid sequences deriving from at least two Termamyl-like α -amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α -amylase, or from at least one Termamyl-like and at least one fungal α -amylase. The Termamyl-like α -amylase from which a partial amino acid sequence
15 derives may, e.g., be any of those specific Termamyl-like α -amylase referred to herein.

For instance, the parent α -amylase may comprise a C-terminal part of an α -amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α -amylase derived from a strain of
20 *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α -amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α -amylase. A such hybrid Termamyl-like α -amylase may be identical to the *Bacillus licheniformis* α -amylase shown
25 in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) is replaced with the N-terminal 33 amino acid residues of the mature protein of the *Bacillus amyloliquefaciens* α -amylase (BAN) shown in SEQ ID NO: 5. A such hybrid may also consist of an amino acid segment corresponding
30 to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α -amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α -amylase

having the amino acid sequence shown in SEQ ID NO: 4.

The non-Termamyl-like α -amylase may, e.g., be a fungal α -amylase, a mammalian or a plant α -amylase or a bacterial α -amylase (different from a Termamyl-like α -amylase). Specific
5 examples of such α -amylases include the *Aspergillus oryzae* TAKA α -amylase, the *A. niger* acid α -amylase, the *Bacillus subtilis* α -amylase, the porcine pancreatic α -amylase and a barley α -amylase. All of these α -amylases have elucidated structures which are markedly different from the structure of a typical
10 Termamyl-like α -amylase as referred to herein.

The fungal α -amylases mentioned above, i.e. derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α -amylases. The fungal α -amylase derived from *Aspergillus*
15 *oryzae* is commercially available under the tradename Fungamyl™.

Furthermore, when a particular variant of a Termamyl-like α -amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g., deletion or substitution) of specific amino acid residues in the
20 amino acid sequence of a specific Termamyl-like α -amylase, it is to be understood that variants of another Termamyl-like α -amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

25 A preferred embodiment of a variant of the invention is one derived from a *B. licheniformis* α -amylase (as parent Termamyl-like α -amylase), e.g., one of those referred to above, such as the *B. licheniformis* α -amylase having the amino acid sequence shown in SEQ ID NO: 4.

30

Altered properties of variants of the invention

The following discusses the relationship between alterations/mutations which may be present in variants of the invention, and desirable alterations in properties (relative to

those a parent, Termamyl-like α -amylase) which may result therefrom.

Increased stability at acidic pH and/or low calcium
5 concentration at high temperatures

The present invention relates to a variant of a parent Termamyl-like α -amylase, which variant α -amylase has been altered in comparison to the parent α -amylase in one or more solvent exposed amino acid residues on the surface of the α -
10 amylase to increase the overall hydrophobicity of the α -amylase and/or to increase the overall numbers of methyl groups in the sidechains of said solvent exposed amino acid residues on the surface.

In a preferred embodiment one or more solvent exposed amino
15 acid residues on a concave surface with inwards bend are altered to more hydrophobic amino acid residues.

In another preferred embodiment one or more solvent exposed amino acid residues on a convex surface are altered to increase the number of methyl groups in the sidechain.

20 The present invention relates to an α -amylase variant of a parent Termamyl-like α -amylase, comprising an alteration at one or more positions selected from the group of:

E376, S417, A420, S356, Y358;

wherein (a) the alteration(s) are independently

25 (i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the
30 position with a different amino acid,

(b) the variant has α -amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like α -amylase having the amino acid sequence of SEQ ID NO: 4.

35 In an embodiment the alteration is one of the following

substitutions:

E376A,R,D,C,Q,G,H,I,K,L,M,N,F,P,S,T,W,Y,V.

In a preferred embodiment the substitution is: E376K.

In an embodiment the alteration is one of the following
5 substitutions: S417A,R,D,C,E,Q,G,H,I,K,L,M,N,F,P,T,W,Y,V;

In a preferred embodiment the substitution is S417T.

In an embodiment the alteration is one of the following
substitutions A420R,D,C,E,Q,G,H,I,K,L,M,N,F,P,S,T,W,Y,V;

In a preferred embodiment the substitution is: A420Q,R.

10 In an embodiment the alteration is one of the following
substitutions: S356A,R,D,C,E,Q,G,H,I,K,L,M,N,F,P,T,W,Y,V.

In an embodiment the alteration is one of the following
substitutions Y358A,R,D,C,E,Q,G,H,I,K,L,M,N,F,P,S,T,W,V.

In a preferred embodiment the substitution is Y358F.

15 In an embodiment of the invention a variant comprises one or
more of the following substitutions: E376K, S417T, A420Q,R,
S356A, Y358F.

The increase in stability at acidic pH and/or low calcium
concentration at high temperatures may be determined using the
20 method described below in Example 2 illustrating the invention.

The parent Termamyl-like α -amylase used as the backbone for
preparing variants of the invention may be any Termamyl-like α -
amylases as defined above.

Specifically contemplated are parent Termamyl-like α -
25 amylases selected from the group derived from *B. licheniformis*,
such as *B. licheniformis* strain ATCC 27811, *B. amyloliquefaciens*,
B. stearothermophilus, *Bacillus* sp. NCIB 12289, NCIB
12512, NCIB 12513 or DSM 9375, and the parent Termamyl-like α -
amylases depicted in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 and 8.

30 In an embodiment of the invention the parent Termamyl-like
 α -amylase is a hybrid α -amylase being identical to the *Bacillus*
licheniformis α -amylase shown in SEQ ID NO: 4 (Termamyl), except
that the N-terminal 35 amino acid residues (of the mature
protein) is replaced with the N-terminal 33 amino acid residues
35 of the mature protein of the *Bacillus amyloliquefaciens* α -
amylase (BAN) shown in SEQ ID NO: 5. The parent Termamyl-like

hybrid α -amylase may be the above mentioned hybrid Termamyl-like α -amylase which further has the following mutations: H156Y+181T+190F+209V+264S (using the numbering in SEQ ID NO: 4). Said backbone is referred to below as "LE174".

5 The parent α -amylase may advantageously further have a mutation in one or more of the following positions: K176, I201 and H205 (using the numbering in SEQ ID NO: 4), especially one or more the following substitutions: K176R, I201F, and H205N (using the numbering in SEQ ID NO: 4), such as specifically the
10 following substitutions: K176R+I201F+H205N (using the numbering in SEQ ID NO: 4).

The inventors have found that the above mentioned variants have increased stability at pHs below 7.0 (i.e., acidic pH) and/or at calcium concentration below 1mM (40ppm) (i.e., low
15 calcium concentrations) at temperatures in the range from 95 to 160°C (i.e., high temperatures) relative to the parent Termamyl-like α -amylase.

Alterations (e.g., by substitution) of one or more solvent exposed amino acid residues which 1) increase the overall
20 hydrophobicity of the enzyme, or 2) increase the number of methyl groups in the sidechains of the solvent exposed amino acid residues improve the temperature stability. It is preferred to alter (e.g., by substitution) to more hydrophobic residues on a concav surface with inwards bend. On a convex surface
25 alterations (e.g., by substitution) to amino acid residues with an increased number of methyl groups in the sidechain are preferred.

Using the program CAST found on the internet at <http://sunrise.cbs.umn.edu/cast/> version 1.0 (release Feb.
30 1998), (reference: Jie Liang, Herbert Edelsbrunner, and Clare Woodward. 1998. Anatomy of protein Pockets and Cavities: Measurements of binding site geometry and implications for ligand design. Protein Science, 7, pp. 1884-1897), a concave area which access to the surface can be identified. Access to
35 the surface is in the program defined as a probe with a diameter of 1.4Å can pass in and out. Using default parameters in the

CAST program concave cavities can be found using the Calcium depleted alpha-amylase structure from *B. licheniformis* as found in the Brookhaven database (1BPL):

Three types of interaction can be rationalised:

- 5 A. Interaction between the sidechain of the residue and the protein,
- B. Interaction between the sidechain of the residue and the surrounding water,
- C. Interaction between the water and the protein.

10 Using the parent Termamyl-like α -amylase shown in SEQ ID NO: 4 as the backbone the following positions are considered to be solvent exposed and may suitably be altered:
E376, S417, A420, S356, Y358.

Corresponding and other solvent exposed positions on the
15 surface of other Termamyl-like α -amylase may be identified using the dssp program by W. Kabsch and C. Sander, Biopolymers 22 (1983) pp. 2577-2637. The convex surfaces can be identified using the AACAVI program part from the WHATIF package (G. Vriend, Whatif and drug design program. J. Mol. Graph. 8, pp.
20 52-56. (1990) version 19980317).

In an embodiment of the invention a variant comprises one or more of the following substitutions: E376K, S417T, A420Q,R, S356A, Y358F.

The inventors have found that the stability at acidic pH
25 and/or low calcium concentration at high temperatures may be increased even more by combining mutations in the above mentioned positions, i.e., E376, S417, A420, S356, Y358, (using the SEQ ID NO: 4 numbering) with mutations in one or more of positions K176, I201, and H205.

30 The following additional substitutions are preferred:

K176A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V;
I201A,R,D,C,E,Q,G,H,L,K,M,N,F,P,S,T,W,Y,V;
H205A,R,D,C,E,Q,G,I,L,K,M,N,F,P,S,T,W,Y,V;

As also shown in Example 2 illustrating the invention
35 combining the following mutations give increased stability:

K176+I201F+H205N+E376K+A420R or

K176+I201F+H205N+S417T+A420Q or

K176+I201F+H205N+S356A+Y358F using the hybrid α -amylase referred to as LE174 as the parent Termamyl-like α -amylase.

5 General mutations in variants of the invention

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues present in the part of the α -amylase variant
10 which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

Analogously, it may be preferred that one or more cysteine
15 residues present among the amino acid residues with which the parent α -amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or leucine.

Furthermore, a variant of the invention may - either as the
20 only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the
25 replacement, in the Termamyl-like α -amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses variants incorporating two or more of the above outlined
30 modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

Cloning a DNA sequence encoding an α -amylase of the invention

The DNA sequence encoding a parent α -amylase may be isolated from any cell or microorganism producing the α -amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using
5 chromosomal DNA or messenger RNA from the organism that produces the α -amylase to be studied. Then, if the amino acid sequence of the α -amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α -amylase-
10 encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α -amylase gene could be used as a probe to identify α -amylase-encoding clones, using hybridization and washing conditions of lower stringency.

15 Yet another method for identifying α -amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α -amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a
20 substrate for α -amylase, thereby allowing clones expressing the α -amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H.
25 Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and syn-
30 thetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also
35 be prepared by polymerase chain reaction (PCR) using specific

primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

5 Once an α -amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide
10 synthesis. In a specific method, a single-stranded gap of DNA, bridging the α -amylase-encoding sequence, is created in a vector carrying the α -amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then
15 filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. How-
20 ever, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into α -amylase-encoding DNA sequences is described in Nelson and Long (1989).
25 It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction
30 endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent α -amylase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent α -amylase, e.g., wherein the variant exhibits altered or increased thermal stability relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent α -amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing an α -amylase variant which has an altered property (i.e. thermal stability) relative to the parent α -amylase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the alpha-amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α -amylase is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cerevisiae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the α -amylase by, e.g., transforming a plasmid
5 containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently
10 present in a genomic or cDNA library prepared from an organism expressing the parent alpha-amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or other-wise exposed to the mutagenising agent. The DNA to be
15 mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a
20 cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently
25 preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram-negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

20 Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent α -amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

Alternative methods of providing α -amylase variants

Alternative methods for providing variants of the invention include gene shuffling method known in the art including the methods, e.g., described in WO 95/22625 (from Affymax Technologies N.V.) and WO 96/00343 (from Novo Nordisk A/S).

Expression of α -amylase variants of the invention

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α -amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α -amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA*

promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α -amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α -amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced

by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α -amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α -amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

10 In a yet further aspect, the present invention relates to a method of producing an α -amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

15 The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α -amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in
20 catalogues of the American Type Culture Collection).

The α -amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous
25 components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

30 Industrial Applications

The α -amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. An enzyme variant of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning detergent
35 compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for

textile desizing. Conditions for conventional starch- conversion processes, including starch liquefaction and/or saccharification processes, are described in, e.g., US 3,912,590 and in EP patent publications Nos. 252,730 and 63,909.

5

Production of sweeteners from starch:

A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes, viz. a liquefaction process followed by a sacchari-
10 fication process and an isomerization process. During the liquefaction process, starch is degraded to dextrins by an α -amylase (e.g. Termamyl™) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2 hours. In order to ensure an optimal enzyme stability under these condi-
15 tions, 1 mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrins are converted into dextrose by addition of a glucoamylase (e.g. AMG™) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g. Promozyme™). Before this step the pH is reduced to a value below
20 4.5, maintaining the high temperature (above 95°C), and the liquefying α -amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

After the saccharification process the pH is increased to a
25 value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized gluco-seisomerase (such as Sweetzyme™).

At least 1 enzymatic improvements of this process could be
30 envisaged. Reduction of the calcium dependency of the liquefying α -amylase. Addition of free calcium is required to ensure adequately high stability of the α -amylase, but free calcium strongly inhibits the activity of the glucoseisomerase and needs to be removed, by means of an expensive unit
35 operation, to an extent which reduces the level of free calcium to below 3-5 ppm. Cost savings could be obtained if such an

operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α -amylase which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α -amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α -amylase.

α -amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α -amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

MATERIALS AND METHODS

Enzymes:

LE174 hybrid alpha-amylase variant: LE174 is a hybrid Termamyl-like alpha-amylase being identical to the Termamyl sequence, i.e., the *Bacillus licheniformis* α -amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the *Bacillus amyloliquefaciens*

alpha-amylase shown in SEQ ID NO: 5, which further have following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

5 Construction of pSNK101

This *E. coli*/Bacillus shuttle vector can be used to introduce mutations without expression of α -amylase in *E. coli* and then be modified in such way that the α -amylase is active in Bacillus. The vector was constructed as follows: The α -amylase gene in the pX vector (pDN1528 with the following alterations within amyL: BAN(1-33), H156Y, A181T, N190F, A209V, Q264S, the plasmid pDN1528 is further described in Example 1) was inactivated by interruption in the PstI site in the 5' coding region of the alpha-amylase gene by a 1.2 kb fragment containing an *E. coli* origin fragment. This fragment was amplified from the pUC19 (GenBank Accession #:X02514) using the forward primer 1: 5'-gacctgcagtcaggcaacta-3' (SEQ ID NO: 28) and the reverse primer 1: 5'-tagagtcgacctgcagggcat-3' (SEQ ID NO: 29). The PCR amplicon and the pX plasmid containing the α -amylase gene were digested with PstI at 37°C for 2 hours. The pX vector fragment and the *E. coli* origin amplicon were ligated at room temperature. for 1 hour and transformed in *E. coli* by electrotransformation. The resulting vector is designated pSnK101.

This *E. coli*/Bacillus shuttle vector can be used to introduce mutations without expression of α -amylase in *E. coli* and then be modified in such way that the α -amylase is active in Bacillus. The vector was constructed as follows: The α -amylase gene in the pX vector (pDN1528 with the following alterations within amyL: BAN(1-33), H156Y+A181T+N190F+A209V+Q264S, the plasmid pDN1528 is further described in Example 1) was inactivated by interruption in the PstI site in the 5' coding region of the alpha-amylase gene by a 1.2 kb fragment containing an *E. coli* origin fragment. This fragment was amplified from the pUC19 (GenBank Accession

#:X02514) using the forward primer 2: 5'-gacctgcagtcaggcaacta-3' (SEQ ID NO: 30) and the reverse primer 2: 5'-tagagtcgacctgcaggcat-3' (SEQ ID NO: 31). The PCR amplicon and the pX plasmid containing the α -amylase gene were digested with PstI at 37°C for 2 hours. The pX vector fragment and the *E. coli* origin amplicon were ligated at room temperature for 1 hour and transformed in *E. coli* by electrotransformation. The resulting vector is designated pSnK101.

10 Low pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 µg/ml chloramphenicol at 37°C for at least 21 hrs. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 90°C for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on assay plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at 50°C. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Secondary screening

Positive transformants after rescreening are picked from the storage plate and tested in a secondary plate assay.

Positive transformants are grown for 22 hours at 37°C in 5 ml LB + chloramphenicol. The *Bacillus* culture of each positive transformant and a control LE174 variant were incubated in citrate buffer, pH 4.5 at 90°C and samples were taken at 5 0,10,20,30,40,60 and 80 minutes. A 3 microliter sample was spotted on a assay plate. The assay plate was stained with 10% Lugol solution. Improved variants were seen as variants with higher residual activity detected as halos on the assay plate than the backbone. The improved variants are determined by 10 nucleotide sequencing.

Fermentation and purification of α -amylase variants

A *B. subtilis* strain harbouring the relevant expression plasmid is streaked on a LB-agar plate with 15 µg/ml 15 chloramphenicol from -80°C stock, and grown overnight at 37°C. The colonies are transferred to 100 ml BPX media supplemented with 15 µg/ml chloramphenicol in a 500 ml shaking flask.

Composition of BPX medium:

	Potato starch	100 g/l
20	Barley flour	50 g/l
	BAN 5000 SKB	0.1 g/l
	Sodium caseinate	10 g/l
	Soy Bean Meal	20 g/l
	Na ₂ HPO ₄ , 12 H ₂ O	9 g/l
25	Pluronic™	0.1 g/l

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear 30 solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 35 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions

which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.

5

Stability determination

All the stability trials are made using the same set up. The method is:

The enzyme is incubated under the relevant conditions (1-
10 4). Samples are taken at 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity is measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

15 The activity measured before incubation (0 minutes) is used as reference (100%). The decline in percent is calculated as a function of the incubation time. The table shows the residual activity after 30 minutes of incubation.

20 Activity determination - (KNU)

One Kilo alph-amyase Unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch (Merck, Amylum Solubile, Erg. B 6, Batch 9947275) per hour in Novo Nordisk's standard method for determination of alpha-amyase based upon the following
25 condition:

Substrate	soluble starch
Calcium content in solvent	0.0043 M
Reaction time	7-20 minutes
Temperature	37°C
30 pH	5.6

Detailed description of Novo Nordisk's analytical method (AF 9) is available on request.

Specific activity determination

35 Assay for α -Amylase Activity

α -amyase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets

(Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

5 For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl_2 , pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The
10 α -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α -amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at
15 620 nm, is a function of the α -amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and
20 absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α -amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour
25 intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α -amylase protein) of the α -amylase in question under the given set of conditions.

30 EXAMPLES

Example 1.

Construction, by random mutagenesis, of Termamyl-like LE174 α -amylase variants having an improved stability at low pH and a reduced dependency on calcium ions for stability compared to the parent enzyme.

5

Random mutagenesis

To improve the stability at low pH and low calcium concentration of the parent LE174 α -amylase variant random mutagenesis in preselected regions was performed.

10 The regions were:

Region:	Residue:
SERI	A425-Y438
SERII	W411-L424
SERIII	G397-G410
15 SERV	T369-H382
SERVII	G310-F323
SERIX	L346-P359

For each six region, random oligonucleotides are synthesized using the same mutation rate (97 % backbone and 1% of each of the three remaining nucleotides giving 3% mutations) in each nucleotide position in the above regions, e.g., 1. position in codon for A425: 97%C, 1%A, 1%T, 1%G. The six random oligonucleotides and if used complementary SOE helping primers are shown in tables 1-6: with the four distribution of 25 nucleotides below.

Table 1.

RSERI: 5'-GC GTT TTG CCG GCC GAC ATA 312 234 322 243 333 133
444 233 423 242 212 211 243 343 CAA ACC TGA ATT-3' (SEQ ID NO:
30 15)

Table 2.

RSERII: 5'-GC GTT TTG CCG GCC GAC ATA CAT TCG CTT TGC CCC ACC
GGG TCC GTC TGT TAT TAA TGC CGC 311 133 241 122 243 113 341 432
35 423 433 223 332 242 331 GCC GAC AAT GTC ATG GTG-3' (SEQ ID NO:
16)

Table 3.

RSERIII: 5'-GTC GCC TTC CCT TGT CCA 433 413 112 423 124 424 423
 411 121 123 124 324 243 233 GTA CGC ATA CTG TTT TCT-3' (SEQ ID
 5 NO: 17)
 Helping primer FSERIII: 5'-TGG ACA AGG GAA GGC GAC AG-3' (SEQ
 ID NO: 18)

Table 4.

10 RSEV: 5-TAA GAT CGG TTC AAT TTT 424 222 311 443 144 112 223
 434 324 441 423 233 222 342 CCC GTA CAT ATC CCC GTA GAA-3 (SEQ
 ID NO: 19)
 Helping primer FSEV: 5-AAA ATT GAA CCG ATC TTA-3 (SEQ ID NO:
 20)

15

Table 5.

FSERVII: 5'-TT CCA TGC TGC ATC GAC ACA GGG AGG CGG CTA TGA TAT
 GAG GAA ATT GCT GAA 344 213 442 342 223 311 431 233 422 411 123
 442 213 122 TGT CGA TAA CCA-3' (SEQ ID NO: 21)
 20
 Helping primer RSEV: 5'- TGT CGA TGC AGC ATG GAA - 3' (SEQ
 ID NO: 22)

Table 6.

25 FSERIX: 5'-GT CCA AAC ATG GTT TAA GCC 432 243 221 343 222 212
 232 313 114 441 123 244 121 333 TCA GGT TTT CTA CGG GGA-3' (SEQ
 ID NO: 23)
 Helping primer RSEV: 5'-GGC TTA AAC CAT GTT TGG AC-3' (SEQ ID
 NO: 24)

30

Distribution of nucleotides in each mutated nucleotide position
 1:97%A, 1%T, 1%C, 1%G
 2:97%T, 1%A, 1%C, 1%G
 3:97%C, 1%A, 1%T, 1%G
 35 4:97%G, 1%A, 1%T, 1%C

Construction of plasmid libraries

Two approximately 1.4 kb fragments were PCR amplified using the primer 1B: 5'-CGA TTG CTG ACG CTG TTA TTT GCG-3' and the random oligonucleotide apparent from table 1, respectively the random oligonucleotide apparent from table 2. The vector pSnK101 and the PCR fragments were digested with EcoRV and EagI for 2 hours. The approximately 3.6 kb vector fragment and the approximately 1.3 kb PCR fragments was purified and ligated overnight and transformed in to *E.coli* and then further transformed into a *Bacillus* host strain as described below. The random oligonucleotides apparent from Tables 3-6 (which by a common term is designated aSER and bSER in Fig. 2) for each region and specific *B. licheniformis* primers 1B (SEQ ID NO: 26) and #63: 5'-CTA TCT TTG AAC ATA AAT TGA AAC C-3' (SEQ ID NO: 27) covering the EcoRV and the EagI sites in the LE174 sequence are used to generate PCR-library-fragments by the overlap extension method (Horton et al., Gene, 77 (1989), pp. 61-68) Figure 2 shows the PCR strategy. The PCR fragments are cloned in the *E. coli*/*Bacillus* shuttle vector pSNK101 (see Materials and Methods) enabling mutagenesis in *E. coli* and immediate expression in *Bacillus subtilis* preventing lethal accumulation of amylases in *E. coli*. After establishing the cloned PCR fragments in *E. coli*, a modified pUC19 fragment is digested out of the plasmid and the promoter and the mutated Termamyl gene is physically connected and expression can take place in the *Bacillus* host .

Screening

The six libraries were screened in the low pH filter assays described in the "Material and Methods" section above.

All variants listed in the table in Example 2 below was prepared as described in Example 1.

EXAMPLE 2

Measurement of stability

Normally, industrial liquefaction processes is run at pH 6.0-6.2 with addition of about 40 ppm free calcium in order to

improve the stability at 95°C-105°C. Variants of the invention have been made in order to improve the stability at

1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40ppm free calcium.

5 An assay which measures the stability at acidic pH, pH 5.0, in the presence of 5ppm free calcium was used to measure the increase in stability.

10 10 µg of the variant was incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.0, containing 5ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.

Results:

15 Increased stability at pH 5.0, 5 ppm calcium incubated at 95°C

MINUTES OF INCUBATION	LE174 WITH K176R+ I201F+ H205N	LE174 WITH K176R+ I201F+ H205N+ E376K+ A420R	LE174 WITH K176R+ I201F+ H205N+ S417T+ A420Q	LE174 WITH K176R+ I201F+ H205N+ S356A+ Y358F
0	100	100	100	100
5	65	61	66	66
10	58	53	60	59
15	51	48	55	56
30	36	39	45	49

Specific activity determination.

The specific activity was determined using the Phadebas assay (Pharmacia) (described above) as activity/mg enzyme. The
20 activity was determined using the α-amylase assay described in the Materials and Methods section herein.

LE174 with the following substitutions:

K176R+I201F+H205N

Specific activity determined: 13400NU/mg

LE174 with the following substitutions:

5 K176R+I201F+H205N+E376K+A420R:

Specific activity determined: 14770NU/mg

LE174 with the following substitutions:

K176R+I201F+H205N+S417T+A420Q:

10 Specific activity determined: 16670NU/mg

LE174 with the following substitutions:

K176R+I201F+H205N+S356A+Y358F:

Specific activity determined: 15300NU/mg

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CLAIMS

1. A variant of a parent Termamyl-like α -amylase, which variant α -amylase has been altered in comparison to the parent α -amylase in one or more solvent exposed amino acid residues on the surface of the α -amylase to increase the overall hydrophobicity of the α -amylase and/or to increase the overall numbers of methyl groups in the sidechains of said solvent exposed amino acid residues on the surface.
2. The variant according to claim 1, wherein one or more solvent exposed amino acid residues on a concav surface with inwards bend are altered to more hydrophobic amino acid residues.
3. The variant according to claim 1, wherein one or more solvent exposed amino acid residues on a convex surface are altered to increase the number of methyl groups in the sidechain.
4. A variant of a parent Termamyl-like α -amylase, comprising an alteration at one or more positions selected from the group of: E376, S417, A420, S356, Y358; wherein (a) the alteration(s) are independently
- (i) an insertion of an amino acid downstream of the amino acid which occupies the position,
 - (ii) a deletion of the amino acid which occupies the position, or
 - (iii) a substitution of the amino acid which occupies the position with a different amino acid,
- (b) the variant has α -amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like α -amylase having the amino acid sequence of SEQ ID NO: 4.
5. The variant according to claim 4, which variant has an alteration in one or more solvent exposed amino acid residues as defined in any of claims 1-3.

6. The variant of any of claims 1-5, wherein the parent Termamyl-like α -amylase is derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. stearothermophilus*, *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375.

7. The variant according to claim 6, wherein the parent α -amylase is derived from *B. licheniformis* strain ATCC 27811.

8. The variant according to claims 1-6, wherein the parent Termamyl-like α -amylase is any of the α -amylases selected from the group depicted in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, and 8.

9. The variant according to any of claims 1-8, wherein the parent Termamyl-like α -amylase has an amino acid sequence which has a degree of identity to SEQ ID NO: 4 of at least 65%, preferably 70%, more preferably at least 80%, even more preferably at least about 90%, even more preferably at least 95%, even more preferably at least 97%, and even more preferably at least 97%.

10. The variant according to any of claims 1-10, wherein the parent Termamyl-like α -amylase is encoded by a nucleic acid sequence which hybridizes under medium, preferred high stringency conditions, with the nucleic acid sequence of SEQ ID NO: 12.

11. The variant according to claims 1-10, wherein the parent Termamyl-like α -amylase is a hybrid of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4 and *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5.

12. The variant according to claim 11, wherein the parent hybrid Termamyl-like α -amylase is LE174.

13. The variant according to any of claims 1-12, wherein the parent α -amylase further has a mutation in one or more of the following positions: K176, I201 and H205 (using the numbering in SEQ ID NO: 4).
- 5 14. The variant according to claim 13, wherein the parent α -amylase has one or more the following substitutions: K176R, I201F and/or H205N (using the numbering in SEQ ID NO: 4).
- 10 15. The variant according to claim 14, wherein the parent α -amylase has the following substitutions: K176R+I201F+H205N (using the numbering in SEQ ID NO: 4).
16. The variant according to claims 1 to 15, wherein the variant
15 has increased stability at pHs below 7.0 (acidic pH) and/or at low calcium concentration and/or at temperatures in the range from 95 to 160°C (high temperatures) relative to the parent α -amylase.
- 20 17. The variant according to any of claims 1 to 16, which variant has one or more of the following substitutions: E376K, S417T, A420Q, R, S356A, Y358F.
18. A DNA construct comprising a DNA sequence encoding an α -
25 amylase variant according to any one of claims 1 to 17.
19. A recombinant expression vector which carries a DNA construct according to claim 18.
- 30 20. A cell which is transformed with a DNA construct according to claim 18 or a vector according to claim 19.
21. A cell according to claim 20, which is a microorganism.
- 35 22. A cell according to claim 21, which is a bacterium or a fungus.

23. The cell according to claim 22, which is a grampositive bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*,
5 *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thuringiensis*.

24. A detergent additive comprising an α -amylase variant according to any one of claims 1 to 17, optionally in the form of a
10 non-dusting granulate, stabilised liquid or protected enzyme.

25. A detergent additive according to claim 24 which contains 0.02-200 mg of enzyme protein/g of the additive.

15 26. A detergent additive according to claims 24 or 25, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

20 27. A detergent composition comprising an α -amylase variant according to any of claims 1 to 17.

28. A detergent composition according to claim 27 which additionally comprises another enzyme such as a protease, a lipase,
25 a peroxidase, another amylolytic enzyme and/or a cellulase.

29. A manual or automatic dishwashing detergent composition comprising an α -amylase variant according to any of claims 1 to
30 17.

30. A dishwashing detergent composition according to claim 29 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a
35 cellulase.

31. A manual or automatic laundry washing composition comprising

an α -amylase variant according to any of claims 1 to 17.

32. A laundry washing composition according to claim 31, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

33. A composition comprising:

- (i) a mixture of the α -amylase from *B. licheniformis* having the sequence shown in SEQ ID NO: 4 with one or more variants according to any of claims 1 to 17 derived from (as the parent Termamyl-like α -amylase) the *B. stearrowthermophilus* α -amylase having the sequence shown in SEQ ID NO: 3; or
- (ii) a mixture of the α -amylase from *B. stearrowthermophilus* having the sequence shown in SEQ ID NO: 3 with one or more variants according to any of claims 1 to 17 derived from one or more other parent Termamyl-like α -amylases; or
- (iii) a mixture of one or more variants according any of claims 1 to 17 derived from (as the parent Termamyl-like α -amylase) the *B. stearrowthermophilus* α -amylase having the sequence shown in SEQ ID NO: 3 with one or more variants according to the invention derived from one or more other parent Termamyl-like α -amylases.

34. The composition comprising a variant of any of claims 1 to 17 wherein the parent α -amylase is a hybrid alpha-amylase comprising a N-terminal part of the *B. amyloliquefaciens* α -amylase shown in SEQ ID NO: 5 and a C-terminal part of the *B. licheniformis* α -amylase shown in SEQ ID NO: 4.

35. The composition according to claim 34, wherein the parent hybrid Termamyl-like α -amylase is LE174

36. The composition according to claims 35, wherein the parent Termamyl-like α -amylase is LE174 with an alteration in one or more of the following positions: K176, I201 and H205.

37. The composition according to claims 36, wherein the parent Termamyl-like α -amylase is LE174 with one or more of the following substitutions: K176R, I201F and H205N.

5

38. Use of an α -amylase variant according to any one of claims 1 to 17 or a composition according to claims 33 to 37 for washing and/or dishwashing.

10 39. Use of an α -amylase variant according to any one of claims 1 to 17 or a composition according to claims 33 to 37 for textile desizing.

15 40. Use of an α -amylase variant according to any of claims 1 to 17 or a composition according to claims 33 to 37 for starch liquefaction.

41. A method for generating a variant of a parent Termamyl-like α -amylase, which variant exhibits increased stability at high
20 temperatures relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Termamyl-like α -amylase to random mutagenesis,
(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

25 (c) screening for host cells expressing a mutated α -amylase which has increased stability at high temperatures relative to the parent Termamyl-like α -amylase.

1/4

	1				50
1	HHNGTNGTMM	QYFEWHL PND	GNHWNRLRDD	ASNLRNRGIT	AIWIPPAWKG
5 2	..NGTNGTMM	QYFEWYLPND	GNHWNRLRSD	ASNLKDKGIS	AVWIPPAWKG
3	HHNGTNGTMM	QYFEWYLPND	GNHWNRLRDD	AANLKSKGIT	AVWIPPAWKG
4VNGTLM	QYFEWYTPND	GQHWKRLQND	AEHLSDIGIT	AVWIPPAYKG
5	..ANLNGTLM	QYFEWYMPND	GQHWRRLQND	SAYLAEHGIT	AVWIPPAYKG
6	.AAPFNGTMM	QYFEWYLPDD	GTLWTKVANE	ANNLSSLGIT	ALWLPPAYKG
10					
	51				100
1	TSQNDVG YGA	YDLYDLGEFN	QKGTVRTKYG	TRSOLESAIH	ALKNNGVQVY
2	ASQNDVG YGA	YDLYDLGEFN	QKGTIRT KYG	TRNQLQA AVN	ALKSNGIQVY
3	TSQNDVG YGA	YDLYDLGEFN	QKGTVRTKYG	TRNQLQA AVT	SLKNNGIQVY
15 4	LSQSDNG YGP	YDLYDLGEFQ	QKGTVRTKYG	TKSELQDAIG	SLHSRNVQVY
5	TSQADV G YGA	YDLYDLGEFH	QKGTVRTKYG	TKGELQSAIK	SLHSRDIN VY
6	TSRSDVG YGV	YDLYDLGEFN	QKGTVRTKYG	TKAQYLQAIQ	AAHAAGMQVY
	101				150
20 1	GDVVMNHKGG	ADATENVLAV	EVNPNNRNQE	ISGDYTIEAW	TKFDFPGRGN
2	GDVVMNHKGG	ADATEMVR AV	EVNPNNRNQE	VSGEYTIEAW	TKFDFPGRGN
3	GDVVMNHKGG	ADGTEIVNAV	EVNRSNRNQE	TSGEYAIEAW	TKFDFPGRGN
4	GDVVLNHKAG	ADATEDVTAV	EVNPNARNQE	TSEEQIKAW	TDFRFPGRGN
5	GDVVINH KGG	ADATEDVTAV	EVDPADRN RV	ISGEHLIKAW	THFHFPGRGS
25 6	ADV VFDHKGG	ADGTEWVDAV	EVNP SDRNQE	ISGTYQIQAW	TKFDFPGRGN
	151				200
1	TYSDFKWRWY	HFDGVDWDQS	RQFQNRIYKF	RGDGKAWDWE	VDSENGNYDY
2	THSNFKWRWY	HFDGVDWDQS	RKLNNRIYKF	RGDGKGWDWE	VDTENGN YDY
30 3	NHSSF KWRWY	HFDGTDWDQS	RQLQNKIYKF	RGTGKAWDWE	VDTENGN YDY
4	TYSDFKWHWY	HFDGADWDES	RKI.SRIFKF	RGEGKAWDWE	VSENGNYDY
5	TYSDFKWHWY	HFDGTDWDES	RKL.NRIYKF	..QGKAWDWE	VSNENGNYDY
6	TYSSF KWRWY	HFDGVDWDES	RKL.SRIYKF	RGIGKAWDWE	VDTENGN YDY

Fig. 1

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5      201                                     250
1      LMYADVDMDH PEVVNELRRW GEWYTNLNL DGFRIDAVKH IKYSFTRDWL
2      LMYADIDMDH PEVVNELRNW GVWYTNLGL DGFRIDAVKH IKYSFTRDWS
3      LMYADVDMDH PEVIHELNRW GVWYTNLNL DGFRIDAVKH IKYSFTRDWL
4      LMYADVDDYH PDVVAETKKW GIWYANELSL DGFRIDAAKH IKFSFLRDWV
10 5      LMYADIDYDH PDVAAEIKRW GTWYANELQL DGFRIDAVKH IKFSFLRDWV
6      LMYADLDMDH PEVVTELKNW GKWYVNTTNI DGFRIDAVKH IKFSFFPDWL

      251                                     300
1      THVRNATGKE MFAVAEFWKN DLGALENYLN KTNWNHVSVD VPLHYNLYNA
15 2      IHVRSATGKN MFAVAEFWKN DLGALENYLN KTNWNHVSVD VPLHYNFYNA
3      THVRNTTGKP MFAVAEFWKN DLGALENYLN KTSWNHSAFD VPLHYNLYNA
4      QAVRQATGKE MFTVAEYWQN NAGKLENYLN KTSFNQSVFD VPLHFNQAA
5      NHVREKTGKE MFTVAEYWQN DLGALENYLN KTNFNHVSVD VPLHYQFHAA
6      SYVRSQTGKP LFTVGEYWSY DINKLHNYIT KTDGTMSLFD APLHNKFYTA

20      301                                     350
1      SNSGGNYDMA KLLNGTVVQK HPMHAVTFVD NHDSQPGESE ESFVQEWFKP
2      SKSGGNYDMR QIFNGTVVQR HPMHAVTFVD NHDSQPGEAL ESFVEEWFKP
3      SNSGGYYDMR NILNGSVVQK HPTHAVTFVD NHDSQPGEAL ESFVQQWFKP
25 4      SSQGGGYDMR RLLDGTVVSR HPEKAVTFVE NHDTQPGQSL ESTVQTFWFKP
5      STQGGGYDMR KLLNGTVVSK HPLKSVTFVD NHDTQPGQSL ESTVQTFWFKP
6      SKSGGAFDMR TLMTNTLMKD QPTLAVTFVD NHDTEPGQAL QSWVDPWFKP

      351                                     400
30 1      LAYALILTRE QGYPSVFYGD YYGIPTHS.. .VPAMKAKID PILEARQNFA
2      LAYALTILTRE QGYPSVFYGD YYGIPTHG.. .VPAMKSKID PILEARQKYA
3      LAYALVLTRE QGYPSVFYGD YYGIPTHG.. .VPAMKSKID PLLQARQTF
4      LAYAFILTRE SGYPQVFYGD MYGTKGTSPK EIPSLKDNIE PILKARKEYA
5      LAYAFILTRE SGYPQVFYGD MYGTKGDSQR EIPALKHKIE PILKARKQYA
35 6      LAYAFILTRQ EGYPCVFYGD YYGIPQYN.. .IPSLKSKID PLLIARRDYA

      401                                     450
1      YGTQHDYFDH HNIIGWTREG NTTHPNSGLA TIMSDGPGGE KWMYVGQNK
2      YGRQN.....
40 3      YGTQHDYFDH HDIIGWTREG NSSHPNSGLA TIMSDGPGGN KWMYVGKNKA
4      YGPQHDIYDH PDVIGWTREG DSSAAKSGLA ALITDGPGGS KRMVAGLKNA
5      YGAQHDIYFDH HDIVGWTREG DSSVANSGLA ALITDGPGGA KRMVVGQRNA
6      YGTQHDYLDH SDIIGWTREG GTEKPGSGLA ALITDGPGGS KWMYVGKQHA

```

Fig. 1 (continued)

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      451                                     500
5  1  GQVWHDITGN KPGTVTINAD GWANFSVNGG SVSIWVKR.. .....
    2  .....
    3  GQVWRDITGN RTGTVTINAD GWGNFSVNGG SVSVWVKQ.. .....
    4  GETWYDITGN RSDTVKIGSD GWGEFHVNDG SVSIYVQ... .....
    5  GETWHDITGN RSEPVINSE GWGEFHVNGG SVSIYVQR.. .....
10  6  GKVFDYLTGN RSDTVTINSD GWGEFKVNGG SVSVWVPRKT TVSTIARPIT

      501                                     519
    1  .....
    2  .....
15  3  .....
    4  .....
    5  .....
    6  TRPWTGEFVR WTEPRLVAW
```

Fig. 1 (continued)

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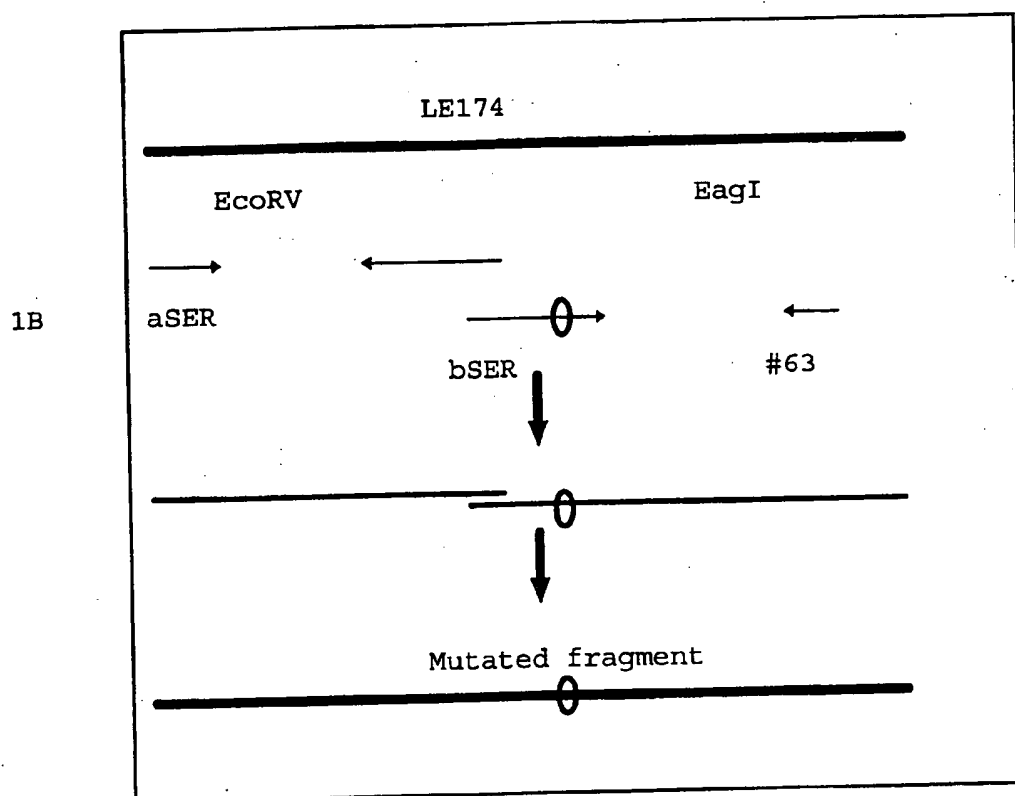


Fig. 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

5 (A) NAME: NOVO NORDISK A/S
 (B) STREET: Novo Alle
 (C) CITY: DK-2880 Bagsvaerd
 (E) COUNTRY: Denmark
 (F) POSTAL CODE (ZIP): DK-2880
 10 (G) TELEPHONE: +45 44 44 88 88
 (H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: α -amylase variants

(iii) NUMBER OF SEQUENCES: 32

(iv) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) Organism: Bacillus sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

30 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
 20 25 30
 35 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45
 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 40 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 45 85 90 95
 Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 50 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
 115 120 125
 Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140
 55 Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
 60 165 170 175
 Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190
 65 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 195 200 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220
 5 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
 245 250 255
 10 Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
 275 280 285
 15 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
 305 310 315 320
 20 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 25 Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
 340 345 350
 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 30 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
 370 375 380
 35 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
 385 390 395 400
 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 40 Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
 435 440 445
 45 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460
 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 Val Trp Val Lys Gln
 485

- 55 (2) INFORMATION FOR SEQ ID NO: 2:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 60 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (iii) Organism: Bacillus sp.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

65 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
 1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
 20 25 30
 Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Ala Trp
 5 35 40 45
 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 10 65 70 75 80
 Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
 85 90 95
 Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 15 100 105 110
 Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
 20 115 120 125
 Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
 130 135 140
 Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
 25 145 150 155 160
 His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
 165 170 175
 Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
 30 180 185 190
 Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
 35 195 200 205
 Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
 210 215 220
 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 40 225 230 235 240
 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
 245 250 255
 Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 45 260 265 270
 Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
 50 275 280 285
 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
 290 295 300
 Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
 55 305 310 315 320
 His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
 60 340 345 350
 Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 65 355 360 365
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala

370 375 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
385 390 395 400

5 Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415

10 Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
435 440 445

15 Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

20 Ile Trp Val Lys Arg
485

(2) INFORMATION FOR SEQ ID NO: 3:

- 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 514 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: protein
 (iii) Organism: *Bacillus stearothermophilus*.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30

40 Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60

45 Leu Gly Glu Phe Asn Gln Lys Gly Ala Val Arg Thr Lys Tyr Gly Thr
65 70 75 80

50 Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110

55 Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140

60 Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160

65 Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
165 170 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu

5

	180	185	190
	Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His		
	195	200	205
5	Pro Glu Val Val Thr Glu Leu Lys Ser Trp Gly Lys Trp Tyr Val Asn		
	210	215	220
10	Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys		
	225	230	235
	Phe Ser Phe Phe Pro Asp Trp Leu Ser Asp Val Arg Ser Gln Thr Gly		
	245	250	255
15	Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys		
	260	265	270
	Leu His Asn Tyr Ile Met Lys Thr Asn Gly Thr Met Ser Leu Phe Asp		
	275	280	285
20	Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Thr		
	290	295	300
	Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro		
	305	310	315
25	Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln		
	325	330	335
30	Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala		
	340	345	350
	Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp		
	355	360	365
35	Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile		
	370	375	380
40	Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His		
	385	390	395
	Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val		
	405	410	415
45	Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro		
	420	425	430
	Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val		
	435	440	445
50	Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser		
	450	455	460
	Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp		
	465	470	475
55	Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Trp Ser Ile Thr Thr		
	485	490	495
60	Arg Pro Trp Thr Asp Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val		
	500	505	510
	Ala Trp		

65

(2) INFORMATION FOR SEQ ID NO: 4:
 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 483 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein
 (iii) Organism: *Bacillus licheniformis*
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10	Ala	Asn	Leu	Asn	Gly	Thr	Leu	Met	Gln	Tyr	Phe	Glu	Trp	Tyr	Met	Pro
	1			5					10						15	
	Asn	Asp	Gly	Gln	His	Trp	Arg	Arg	Leu	Gln	Asn	Asp	Ser	Ala	Tyr	Leu
			20						25					30		
15	Ala	Glu	His	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Tyr	Lys	Gly
			35					40					45			
	Thr	Ser	Gln	Ala	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr	Asp	Leu
		50					55					60				
20	Gly	Glu	Phe	His	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly	Thr	Lys
	65				70					75					80	
	Gly	Glu	Leu	Gln	Ser	Ala	Ile	Lys	Ser	Leu	His	Ser	Arg	Asp	Ile	Asn
25				85						90					95	
	Val	Tyr	Gly	Asp	Val	Val	Ile	Asn	His	Lys	Gly	Gly	Ala	Asp	Ala	Thr
			100						105					110		
30	Glu	Asp	Val	Thr	Ala	Val	Glu	Val	Asp	Pro	Ala	Asp	Arg	Asn	Arg	Val
			115					120					125			
	Ile	Ser	Gly	Glu	His	Leu	Ile	Lys	Ala	Trp	Thr	His	Phe	His	Phe	Pro
35		130					135				140					
	Gly	Arg	Gly	Ser	Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp	Tyr	His	Phe
	145					150				155					160	
	Asp	Gly	Thr	Asp	Trp	Asp	Glu	Ser	Arg	Lys	Leu	Asn	Arg	Ile	Tyr	Lys
40				165						170					175	
	Phe	Gln	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Ser	Asn	Glu	Asn	Gly	Asn
			180						185					190		
45	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Tyr	Asp	His	Pro	Asp	Val
			195					200					205			
	Ala	Ala	Glu	Ile	Lys	Arg	Trp	Gly	Thr	Trp	Tyr	Ala	Asn	Glu	Leu	Gln
		210					215					220				
50	Leu	Asp	Gly	Phe	Arg	Leu	Asp	Ala	Val	Lys	His	Ile	Lys	Phe	Ser	Phe
	225					230					235					240
	Leu	Arg	Asp	Trp	Val	Asn	His	Val	Arg	Glu	Lys	Thr	Gly	Lys	Glu	Met
55				245						250					255	
	Phe	Thr	Val	Ala	Glu	Tyr	Trp	Gln	Asn	Asp	Leu	Gly	Ala	Leu	Glu	Asn
			260						265					270		
60	Tyr	Leu	Asn	Lys	Thr	Asn	Phe	Asn	His	Ser	Val	Phe	Asp	Val	Pro	Leu
			275					280					285			
	His	Tyr	Gln	Phe	His	Ala	Ala	Ser	Thr	Gln	Gly	Gly	Gly	Tyr	Asp	Met
		290					295					300				
65	Arg	Lys	Leu	Leu	Asn	Gly	Thr	Val	Val	Ser	Lys	His	Pro	Leu	Lys	Ser
	305					310					315					320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
 325 330 335

5 Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
 340 345 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
 355 360 365

10 Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
 370 375 380

15 Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
 385 390 395 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
 405 410 415

20 Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
 420 425 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
 435 440 445

25 Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
 450 455 460

30 Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
 465 470 475 480

Val Gln Arg

35 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 480 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) Organism: *Bacillus amyloliquefaciens*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

45 Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
 1 5 10 15

Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
 20 25 30

50 Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser
 35 40 45

55 Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu
 50 55 60

Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
 65 70 75 80

60 Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr
 85 90 95

Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
 100 105 110

65 Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser
 115 120 125

	Glu	Glu	Tyr	Gln	Ile	Lys	Ala	Trp	Thr	Asp	Phe	Arg	Phe	Pro	Gly	Arg	
	130						135					140					
5	Gly	Asn	Thr	Tyr	Ser	Asp	Phe	Lys	Trp	His	Trp	Tyr	His	Phe	Asp	Gly	
	145					150					155					160	
	Ala	Asp	Trp	Asp	Glu	Ser	Arg	Lys	Ile	Ser	Arg	Ile	Phe	Lys	Phe	Arg	
					165					170					175		
10	Gly	Glu	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Ser	Ser	Glu	Asn	Gly	Asn	
				180					185					190			
	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Tyr	Asp	His	Pro	Asp	Val	
15			195					200					205				
	Val	Ala	Glu	Thr	Lys	Lys	Trp	Gly	Ile	Trp	Tyr	Ala	Asn	Glu	Leu	Ser	
	210						215					220					
20	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Ala	Lys	His	Ile	Lys	Phe	Ser	Phe	
	225					230					235					240	
	Leu	Arg	Asp	Trp	Val	Gln	Ala	Val	Arg	Gln	Ala	Thr	Gly	Lys	Glu	Met	
					245					250					255		
25	Phe	Thr	Val	Ala	Glu	Tyr	Trp	Gln	Asn	Asn	Ala	Gly	Lys	Leu	Glu	Asn	
				260					265					270			
	Tyr	Leu	Asn	Lys	Thr	Ser	Phe	Asn	Gln	Ser	Val	Phe	Asp	Val	Pro	Leu	
30			275					280					285				
	His	Phe	Asn	Leu	Gln	Ala	Ala	Ser	Ser	Gln	Gly	Gly	Gly	Tyr	Asp	Met	
	290					295					300						
35	Arg	Arg	Leu	Leu	Asp	Gly	Thr	Val	Val	Ser	Arg	His	Pro	Glu	Lys	Ala	
	305					310					315					320	
	Val	Thr	Phe	Val	Glu	Asn	His	Asp	Thr	Gln	Pro	Gly	Gln	Ser	Leu	Glu	
				325						330					335		
40	Ser	Thr	Val	Gln	Thr	Trp	Phe	Lys	Pro	Leu	Ala	Tyr	Ala	Phe	Ile	Leu	
				340					345					350			
	Thr	Arg	Glu	Ser	Gly	Tyr	Pro	Gln	Val	Phe	Tyr	Gly	Asp	Met	Tyr	Gly	
45			355					360					365				
	Thr	Lys	Gly	Thr	Ser	Pro	Lys	Glu	Ile	Pro	Ser	Leu	Lys	Asp	Asn	Ile	
		370					375					380					
50	Glu	Pro	Ile	Leu	Lys	Ala	Arg	Lys	Glu	Tyr	Ala	Tyr	Gly	Pro	Gln	His	
	385					390					395					400	
	Asp	Tyr	Ile	Asp	His	Pro	Asp	Val	Ile	Gly	Trp	Thr	Arg	Glu	Gly	Asp	
					405					410					415		
55	Ser	Ser	Ala	Ala	Lys	Ser	Gly	Leu	Ala	Ala	Leu	Ile	Thr	Asp	Gly	Pro	
				420					425					430			
	Gly	Gly	Ser	Lys	Arg	Met	Tyr	Ala	Gly	Leu	Lys	Asn	Ala	Gly	Glu	Thr	
60			435					440					445				
	Trp	Tyr	Asp	Ile	Thr	Gly	Asn	Arg	Ser	Asp	Thr	Val	Lys	Ile	Gly	Ser	
			450				455					460					
65	Asp	Gly	Trp	Gly	Glu	Phe	His	Val	Asn	Asp	Gly	Ser	Val	Ser	Ile	Tyr	
	465					470					475					480	

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 10 (iii) Organism: Bacillus sp.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10 15
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
 20 25 30
 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45
 Lys Gly Ala Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 Thr Arg Ser Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 85 90 95
 Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
 100 105 110
 Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn
 115 120 125
 Gln Glu Val Thr Gly Glu Tyr Thr Ile Glu Ala Trp Thr Arg Phe Asp
 130 135 140
 Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
 145 150 155 160
 His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Arg Leu Asn Asn Arg
 165 170 175
 Ile Tyr Lys Phe Arg Gly His Gly Lys Ala Trp Asp Trp Glu Val Asp
 180 185 190
 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
 195 200 205
 Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
 210 215 220
 Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
 225 230 235 240
 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
 245 250 255
 Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
 260 265 270
 Gly Ala Ile Glu Asn Tyr Leu Gln Lys Thr Asn Trp Asn His Ser Val
 275 280 285

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Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
 290 295 300
 Gly Asn Tyr Asp Met Arg Asn Ile Phe Asn Gly Thr Val Val Gln Arg
 5 305 310 315 320
 His Pro Ser His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
 325 330 335
 10 Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
 340 345 350
 Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
 355 360 365
 15 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
 370 375 380
 20 Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys
 385 390 395 400
 Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
 405 410 415
 25 Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
 420 425 430
 Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
 435 440 445
 30 Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
 450 455 460
 35 Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
 465 470 475 480
 Ile Trp Val Asn Lys
 485

- 40 (2) INFORMATION FOR SEQ ID NO: 7:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 485 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 45 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (iii) Organism: Bacillus sp.
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

50 His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
 1 5 10
 Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
 20 25 30
 55 Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
 35 40 45
 60 Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
 50 55 60
 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
 65 70 75 80
 65 Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
 85 90 95

	Ile	Gln	Val	Tyr	Gly	Asp	Val	Val	Met	Asn	His	Lys	Gly	Gly	Ala	Asp	
				100					105					110			
5	Gly	Thr	Glu	Ile	Val	Asn	Ala	Val	Glu	Val	Asn	Arg	Ser	Asn	Arg	Asn	
			115					120					125				
	Gln	Glu	Thr	Ser	Gly	Glu	Tyr	Ala	Ile	Glu	Ala	Trp	Thr	Lys	Phe	Asp	
		130					135					140					
10	Phe	Pro	Gly	Arg	Gly	Asn	Asn	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr	
	145					150					155					160	
	His	Phe	Asp	Gly	Thr	Asp	Trp	Asp	Gln	Ser	Arg	Gln	Leu	Gln	Asn	Lys	
					165					170					175		
15	Ile	Tyr	Lys	Phe	Arg	Gly	Thr	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp	
				180					185					190			
20	Thr	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Val	Asp	Met	
			195					200					205				
	Asp	His	Pro	Glu	Val	Ile	His	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr	
		210					215					220					
25	Thr	Asn	Thr	Leu	Asn	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His	
	225					230					235					240	
	Ile	Lys	Tyr	Ser	Phe	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Thr	
					245					250					255		
30	Thr	Gly	Lys	Pro	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu	
				260					265					270			
35	Gly	Ala	Ile	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Ser	Trp	Asn	His	Ser	Val	
			275					280					285				
	Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly	
		290					295					300					
40	Gly	Tyr	Tyr	Asp	Met	Arg	Asn	Ile	Leu	Asn	Gly	Ser	Val	Val	Gln	Lys	
	305					310					315					320	
	His	Pro	Thr	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro	
					325					330					335		
45	Gly	Glu	Ala	Leu	Glu	Ser	Phe	Val	Gln	Gln	Trp	Phe	Lys	Pro	Leu	Ala	
				340					345					350			
50	Tyr	Ala	Leu	Val	Leu	Thr	Arg	Glu	Gln	Gly	Tyr	Pro	Ser	Val	Phe	Tyr	
			355					360					365				
	Gly	Asp	Tyr	Tyr	Gly	Ile	Pro	Thr	His	Gly	Val	Pro	Ala	Met	Lys	Ser	
		370					375					380					
55	Lys	Ile	Asp	Pro	Leu	Leu	Gln	Ala	Arg	Gln	Thr	Phe	Ala	Tyr	Gly	Thr	
	385					390					395					400	
	Gln	His	Asp	Tyr	Phe	Asp	His	His	Asp	Ile	Ile	Gly	Trp	Thr	Arg	Glu	
					405					410					415		
60	Gly	Asn	Ser	Ser	His	Pro	Asn	Ser	Gly	Leu	Ala	Thr	Ile	Met	Ser	Asp	
				420					425					430			
65	Gly	Pro	Gly	Gly	Asn	Lys	Trp	Met	Tyr	Val	Gly	Lys	Asn	Lys	Ala	Gly	
			435					440					445				
	Gln	Val	Trp	Arg	Asp	Ile	Thr	Gly	Asn	Arg	Thr	Gly	Thr	Val	Thr	Ile	

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	260	265	270
	Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val		
	275	280	285
5	Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly		
	290	295	300
10	Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys		
	305	310	315
	His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro		
	325	330	335
15	Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala		
	340	345	350
	Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr		
	355	360	365
20	Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala		
	370	375	380
25	Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr		
	385	390	395
	Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu		
	405	410	415
30	Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp		
	420	425	430
	Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly		
	435	440	445
35	Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile		
	450	455	460
40	Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser		
	465	470	475
	Ile Trp Val Lys Arg		
	485		

45 (2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1455 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) Organism: Bacillus sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

55	CATCATAATG GAACAAATGG TACTATGATG CAATATTTTCG AATGGTATTT GCCAAATGAC	60
	GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120
	GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC	180
60	TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA	240
	ACACGCAACC AGCTACAGGC TCGCGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT	300
65	GGTGTATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA	360
	GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG	420

ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480
 CATTTCGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540
 5 AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600
 CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAACTGG 660
 10 GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT 720
 ATAAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780
 ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840
 15 AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA 900
 TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960
 20 CATCCAAACAC ATGCCGTTAC TTTTGTGGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020
 GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080
 CAAGGTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCGG 1140
 25 GCTATGAAAT CTAAATAGA CCTCTTCTG CAGGCACGTC AAACTTTTCG CTATGGTACG 1200
 CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260
 30 CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320
 TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380
 ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440
 35 GTTTGGGTGA AGCAA 1455

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 1455 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45 (iii) Organism: *Bacillus* sp.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60
 50 GGGAACTACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120
 GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATGGAGCC 180
 TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240
 55 ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300
 GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360
 60 GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420
 ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480
 CATTTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540
 65 CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600

TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660
GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720
5 ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780
ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840
AAAACAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900
10 TCAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAAG 960
CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA 1020
15 GAATCATTTG TACAAGAATG GTTTAAGCCA CTGCTTATG CGCTTATTTT AACAAGAGAA 1080
CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140
GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200
20 CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260
CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320
25 TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380
ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440
ATTTGGGTGA AACGA 1455
30

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 1548 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) Organism: *Bacillus stearothermophilus*
40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCCGCACCGT TTAACGGCAC CATGATGCAG TATTTTGAAT GGTAATTGCC GGATGATGGC 60
ACGTTATGGA CCAAAGTGGC CAATGAAGCC AACAACCTAT CCAGCCTTGG CATCACCGCT 120
45 CTTTGGCTGC CGCCCGCTTA CAAAGGAACA AGCCGACGCG ACGTAGGGTA CGGAGTATAC 180
GACTTGTATG ACCTCGGCGA ATTCAATCAA AAAGGGACCG TCCGCACAAA ATACGGAACA 240
50 AAAGCTCAAT ATCTTCAAGC CATTCAAGCC GCCCAGCCG CTGGAATGCA AGTGTACGCC 300
GATGTCGTGT TCGACCATAA AGGCGGCGCT GACGGCACGG AATGGGTGGA CGCCGTCGAA 360
GTCAATCCGT CCGACCGCAA CCAAGAAATC TCGGGCACCT ATCAAATCCA AGCATGGACG 420
55 AAATTTGATT TTCCCGGGCG GGGCAACACC TACTCCAGCT TTAAGTGGCG CTGGTACCAT 480
TTTGACGGCG TTGATTGGGA CGAAAGCCGA AAATTGAGCC GCATTTACAA ATTCCGCGGC 540
60 ATCGGCAAAG CGTGGGATTG GGAAGTAGAC ACGGAAAACG GAACTATGA CTACTTAATG 600
TATGCCGACC TTGATATGGA TCATCCCGAA GTCGTGACCG AGCTGAAAAA CTGGGGGAAA 660
TGGTATGTCA ACACAACGAA CATTGATGGG TTCCGGCTTG ATGCCGTCAA GCATATTAAG 720
65 TTCAGTTTTT TTCTGATTG GTTGTCTGAT GTGCGTTCTC AGACTGGCAA GCCGCTATTT 780

ACCGTCGGGG AATATTGGAG CTATGACATC AACAAAGTTGC ACAATTACAT TACGAAAACA 840
 GACGGAACGA TGTCTTTGTT TGATGCCCCG TTACACAACA AATTTTATAC CGCTTCCAAA 900
 5 TCAGGGGGCG CATTTGATAT GCGCACGTTA ATGACCAATA CTCTCATGAA AGATCAACCG 960
 ACATTGGCCG TCACCTTCGT TGATAATCAT GACACCGAAC CCGGCCAAGC GCTGCAGTCA 1020
 TGGGTCGACC CATGGTTCAA ACCGTTGGCT TACGCCTTTA TTCTAACTCG GCAGGAAGGA 1080
 10 TACCCGTGCG TCTTTTATGG TGACTATTAT GGCATTCCAC AATATAACAT TCCTTCGCTG 1140
 AAAAGCAAAA TCGATCCGCT CCTCATCGCG CGCAGGGATT ATGCTTACGG AACGCAACAT 1200
 15 GATTATCTTG ATCACTCCGA CATCATCGGG TGGACAAGGG AAGGGGGCAC TGAAAAACCA 1260
 GGATCCGGAC TGGCCGCACT GATCACCGAT GGGCCGGGAG GAAGCAAATG GATGTACGTT 1320
 GGCAAACAAC ACGCTGGAAA AGTGTCTTAT GACCTTACCG GCAACCGGAG TGACACCGTC 1380
 20 ACCATCAACA GTGATGGATG GGGGGAATTC AAAGTCAATG GCGGTTCCGT TTCGGTTTGG 1440
 GTTCCTAGAA AAACGACCGT TTCTACCATC GCTCGGCCGA TCACAACCCG ACCGTGGACT 1500
 25 GGTGAATTCG TCCGTTGGAC CGAACCACGG TTGGTGGCAT GGCCTTGA 1548

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 30 (A) LENGTH: 1920 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 35 (iii) Organism: *Bacillus licheniformis*
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 421..1872
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

40 CGGAAGATTG GAAGTACAAA AATAAGCAAA AGATTGTCAA TCATGTCATG AGCCATGCGG 60
 GAGACGGAAA AATCGTCTTA ATGCACGATA TTTATGCAAC GTTCGCAGAT GCTGCTGAAG 120
 45 AGATTATTAA AAAGCTGAAA GCAAAAGGCT ATCAATTGGT AACTGTATCT CAGCTTGAAG 180
 AAGTGAAGAA GCAGAGAGGC TATTGAATAA ATGAGTAGAA GCGCCATATC GGCGCTTTTC 240
 TTTTGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT TTATACAACA 300
 50 TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAAACG GCTTTACGCC 360
 CGATTGCTGA CGCTGTTATT TGCCTCATC TTCTTGCTGC CTCATTCTGC AGCAGCGGCG 420
 55 GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC 468
 AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA AAC GAC TCG GCA TAT TTG 516
 GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA 564
 60 ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA 612
 GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA 660
 65 GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC 708
 GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC 756

	GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA	804
	ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG	852
5	GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT	900
	GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG	948
10	TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC	996
	TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC	1044
	GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA	1092
15	TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT	1140
	TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG	1188
20	TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC	1236
	TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT	1284
	CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG	1332
25	AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG	1380
	GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG	1428
30	TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC	1476
	ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG	1524
	ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT	1572
35	GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT	1620
	GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC	1668
40	AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC	1716
	GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA	1764
	TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG	1812
45	GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT	1860
	GTT CAA AGA TAG AAGAGCAGAG AGGACGGATT TCCTGAAGGA AATCCGTTTT	1912
50	TTTATTTT	1920

- (2) INFORMATION FOR SEQ ID NO: 13:
- 55 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1455 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- 60 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) Organism: *Bacillus* sp.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

	CATCATAATG GAACAAATGG TACTATGATG CAATATTTTCG AATGGTATTT GCCAAATGAC	60
65	GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA	120

GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC 180
 TATGATTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA 240
 5 ACACGCAACC AGCTACAGGC TGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300
 GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360
 GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420
 10 ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480
 CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540
 15 AGGGGAACAG GCAAGGCCTG GGAAGTGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600
 CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660
 GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT 720
 20 ATAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780
 ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840
 25 AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCTCTCC ACTATAATTT GTACAATGCA 900
 TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960
 CATCCAACAC ATGCCGTTAC TTTTGTGTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020
 30 GAATCCTTTG TTCAACAATG GTTTAAACCA CTTCATATG CATTGGTTCT GACAAGGGAA 1080
 CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCGG 1140
 35 GCTATGAAAT CTAAATAGA CCCTCTTCTG CAGGCACGTC AAACCTTTGC CTATGGTACG 1200
 CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260
 CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320
 40 TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380
 ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440
 45 GTTTGGGTGA AGCAA 1455

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 1455 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic).

(iii) Organism: *Bacillus* sp.

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60
 GGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120
 60 GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAA AATGATGTGGG GTATGGAGCC 180
 TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240
 65 ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300
 GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360

GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420
 ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480
 5 CATTTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540
 CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCCG AAAATGGAAA TTATGATTAT 600
 10 TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660
 GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720
 ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780
 15 ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840
 AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900
 20 TCAAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAAG 960
 CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA 1020
 GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAAGAGAA 1080
 25 CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140
 GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200
 30 CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260
 CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320
 TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380
 35 ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440
 ATTTGGGTGA AACGA 1455

40 (2) INFORMATION FOR SEQ ID NO: 15:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 74 base pairs
 (B) TYPE: nucleic acid
 45 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: other nucleic acid
 (ix) FEATURE:
 (A) NAME/ KEY: misc-feature:
 50 (B) OTHER INFORMATION: /desc = "RSERI"
 (ix) FEATURE:
 (A) NAME/KEY: misc-feature
 (B) LOCATION: 21-62
 (D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
 55 2:97%T, 1%A, 1%C, 1%G
 3:97%C, 1%A, 1%T, 1%G
 4:97%G, 1%A, 1%T, 1%C
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
 GCGTTTTGCC GGCCGACATA 3122343222 4333313344
 60 4233423242 2122112433 43CAAACCTG AATT

74

(2) INFORMATION FOR SEQ ID NO: 16:
 (i) SEQUENCE CHARACTERISTICS:
 65 (A) LENGTH: 122 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/ KEY: misc-feature:

5 (B) OTHER INFORMATION: /desc = "RSERII"

(ix) FEATURE:

(A) NAME/KEY: misc-feature

(B) LOCATION: 63-104

(D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:
15 GCGTTTGGCC GGCCGACATA CATTGCTTT GCCCACC GG GTCCGTCTGT
TATTAATGCC GC31113324 1122243113 3414324234 3322333224
2331GCCGAC AATGTCATGG TG 122

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

25 (ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "RSERIII"

(ix) FEATURE:

(A) NAME/KEY: misc-feature

30 (B) LOCATION: 19-60

(D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:
35 GTCGCCTTCC CTTGTCCA43 3413112423 1244244234 1112112312
4324243233 GTACGCATAC TGT TTTCT 78

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "FSERIII"

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:
TGGACAAGGG AAGGCGACAG 20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 81 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

60 (ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "RSERV"

(ix) FEATURE:

(A) NAME/KEY: misc-feature

65 (B) LOCATION: 19-60

(D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G

3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
TAAGATCGGT TCAATTTT42 4222311443 1441122234 3432444142
5 3233222342 CCCGTACATA TCCCCGTAGA A

(2) INFORMATION FOR SEQ ID NO: 20:
(i) SEQUENCE CHARACTERISTICS:
10 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
15 (ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "FSERV"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
AAAATTGAAC CGATCTTA 18
20

(2) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
25 (A) LENGTH: 107 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
30 (A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "FSERVII"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 54-95
35 (D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
40 TTCCATGCTG CATCGACACA GGGAGGCGGC TATGATATGA GGAAATTGCT
GAA3442134 4234222331 1431233422 4111234422 13122TGTCG
ATAACCA 108

45 (2) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "RSERVII"
55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
TGTCGATGCA GCATGGAA 18

(2) INFORMATION FOR SEQ ID NO: 23:
60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
65 (ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "FSERIX"

(ix) FEATURE:

(A) NAME/KEY: misc-feature

(B) LOCATION: 21-62

5 (D): OTHER INFORMATION: /Note= 1:97%A, 1%T, 1%C, 1%G
2:97%T, 1%A, 1%C, 1%G
3:97%C, 1%A, 1%T, 1%G
4:97%G, 1%A, 1%T, 1%C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

10 GTCCAAACAT GGTTTAAGCC 4322432213 4322221223 2313114441
1232441213 33TCAGGTTT TCTACGGGGA 80

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

20 (ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "RSERIX"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGCTTAAACC ATGTTTGGAC 20

25 (2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/ KEY: misc-feature:

35 (B) OTHER INFORMATION: /desc = "Primer 1B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CGATTGCTGA CGCTGTTATT TGCG 24

(2) INFORMATION FOR SEQ ID NO: 27:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "Primer #63"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

50 CTATCTTTGA ACATAAATTG AAACC 25

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

60 (ix) FEATURE:

(A) NAME/ KEY: misc-feature:

(B) OTHER INFORMATION: /desc = "forward Primer1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

gacctgcagt caggcaacta 20

65 (2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

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      (A) LENGTH: 20 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
5  (ii) MOLECULE TYPE: other nucleic acid
    (ix) FEATURE:
      (A) NAME/ KEY: misc-feature:
      (B) OTHER INFORMATION: /desc = "reverse primer 1"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
10 tagagtcgac ctgcaggcat 20

    (2) INFORMATION FOR SEQ ID NO: 30:
      (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 20 base pairs
15      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
    (ix) FEATURE:
20      (A) NAME/ KEY: misc-feature:
      (B) OTHER INFORMATION: /desc = "forward primer 2"
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
    gacctgcagt caggcaacta 20

25 (2) INFORMATION FOR SEQ ID NO: 31:
      (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 25 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
30      (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: other nucleic acid
    (ix) FEATURE:
      (A) NAME/ KEY: misc-feature:
      (B) OTHER INFORMATION: /desc = "reverse primer 2"
35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
    tagagtcgac ctgcaggcat 20

    (2) INFORMATION FOR SEQ ID NO: 32:
40      (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 2084 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
45      (ii) MOLECULE TYPE: DNA (genomic)
      (iii) Organism: Bacillus amyloliquefaciens
    (ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 343..1794
50      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTTGATGACT GATGATTTGG 60
CTGAAGAAGT GGATCGATTG TTTGAGAAAA GAAGAAGACC ATAAAAATAC CTTGTCTGTC 120
55 ATCAGACAGG GTATTTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAAT AAGGAATAAA 180
GGGGGGTTGT TATTATTTTA CTGATATGTA AAATATAATT TGTATAAGAA AATGAGAGGG 240
60 AGAGGAAACA TGATTCAAAA ACGAAAGCGG ACAGTTTCGT TCAGACTTGT GCTTATGTGC 300
ACGCTGTTAT TTGTCAGTTT GCCGATTACA AAAACATCAG CC GTA AAT GGC ACG 354
CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG 402
65 AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA TCG GAT ATC GGA ATC ACT 450

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	GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA TCC GAT AAC	498
	GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA	546
5	GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG	594
	ATC GGC TCA CTG CAT TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT	642
	TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA GAA GAT GTA ACT GCC GTC	690
10	GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG GAA TAT CAA	738
	ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC	786
15	AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT	834
	GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA	882
	GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC GGC AAC TAT GAC TAT TTA	930
20	ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT GAT GTC GTG GCA GAG ACA	978
	AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC	1026
25	CGT ATT GAT GCC GCC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG	1074
	GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG	1122
	GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC GAA AAC TAC TTG AAT AAA	1170
30	ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT TTC AAT TTA	1218
	CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG CGT TTG CTG	1266
35	GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT	1314
	GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA	1362
	ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT ATT TTG ACA AGA GAA TCC	1410
40	GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA AAA GGG ACA	1458
	TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA	1506
45	AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC	1554
	CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC	1602
	AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC GGA CCC GGC GGA TCA AAG	1650
50	CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG TAT GAC ATA	1698
	ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA	1746
55	GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA	1794
	GGTAATAAAA AAACACCTCC AAGCTGAGTG CGGGTATCAG CTGGAGGTG CGTTTATTTT	1854
	TTTACCCGTA TGACAAGGTC GGCATCAGGT GTGACAAATA CGGTATGCTG GCTGTCATAG	1914
60	GTGACAAATC CGGGTTTTGC GCCGTTTGGC TTTTTCACAT GTCTGATTTT TGTATAATCA	1974
	ACAGGCACGG AGCCGGAATC TTTCGCCTTG GAAAAATAAG CGGCGATCGT AGCTGCTTCC	2034
65	AATATGGATT GTTCATCGGG ATCGCTGCTT TTAATCACAA CGTGGGATCC	2084

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00628

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9510603 A1 (NOVO NORDISK A/S), 20 April 1995 (20.04.95), see claim 14	1-16,18-41
A	--	17
X	WO 9623873 A1 (NOVO NORDISK A/S), 8 August 1996 (08.08.96), page 23, line 24 and forward	41
A	--	1-39
A	WO 9743424 A1 (GENENCOR INTERNATIONAL, INC.), 20 November 1997 (20.11.97), page 12, lines 4-24	1-41
	-- -----	

☐ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 March 2000

Date of mailing of the international search report

06 -04- 2000

Name and mailing address of the ISA/

Swedish Patent Office

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Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00628

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00628

The wording "solvent exposed amino acid residues" of claim 1 may include other (unknown) residues in addition to those given in the present application (c.f. the specification page 16, lines 10-13). Therefore, the search has been incomplete and restricted to those residues specified in the application (c.f. PCT, Article 6).

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.
PCT/DK 99/00628

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9510603 A1	20/04/95	AU 7807494 A	04/05/95
		BR 9407767 A	18/03/97
		CA 2173329 A	20/04/95
		CN 1134725 A	30/10/96
		EP 0722490 A	24/07/96
		FI 961524 A	30/05/96
		JP 9503916 T	22/04/97
		US 5753460 A	19/05/98
WO 9623873 A1	08/08/96	US 5801043 A	01/09/98
		AU 4483396 A	21/08/96
		BR 9607735 A	14/07/98
		CA 2211405 A	08/08/96
		CN 1172500 A	04/02/98
		EP 0815208 A	07/01/98
WO 9743424 A1	20/11/97	JP 11503003 T	23/03/99
		AU 2996997 A	05/12/97
		EP 0927259 A	07/07/99
		US 5763385 A	09/06/98